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Differentiation of embryonic haemopoietic stem cells from mouse blastocysts grown *in vitro*

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Summary

Embryonic haemopoietic stem cells can differentiate from mouse blastocysts grown *in vitro*. Mouse blastocysts were cultured for 3 or 4 days and the resultant cells were injected intravenously into lethally X-irradiated or genetically anaemic recipient mice. Blastocysts grown *in vitro* did not maintain normal embryonic morphology. The presence of donor haemoglobin and donor lymphocytic glucose phosphate isomerase in grafted recipients, demonstrates the presence of embryonic haemopoietic stem cells. Recipients of embryonic haemopoietic stem cells, obtained from growth *in vitro*, were haematologically stable with no evidence of neoplasia.

Pluripotent embryonic cells, maintained on fibroblast feeder layers, were unable to colonize X-irradiated or genetically anaemic mice. Recipients of pluripotent cells died at the same time as saline-injected controls.

Key words: haemopoietic stem cell, mouse blastocyst, fibroblast, haemoglobin, glucose phosphate isomerase.

Introduction

Many workers have attempted to grow mouse and rabbit embryos through the implantation period, *in vitro*, with varying amounts of success (Cole *et al.* 1966; Pienkowski *et al.* 1974; Sherman, 1975; McLaren & Hensleigh, 1975; Wiley & Pedersen, 1977; Hsu, 1979, 1980). Human embryos have also been grown *in vitro* from fertilization to early post-implantation stages (Fishel *et al.* 1984; Lindenberg *et al.* 1985; Lindenberg *et al.* 1986). Only one worker (Hsu, 1979, 1980) has described almost normal differentiation of the mouse blastocyst to early somite stage *in vitro*. Many of these workers described the formation of blood islands, analogous to those found *in vivo*, in the developing embryos. These blood islands may contain haemopoietic stem cells capable of repopulating deficient recipients.

Pluripotent cell lines can be obtained directly from *in vitro* cultures of mouse blastocysts (Evans & Kaufman, 1981; Martin, 1981). Such cells have been called embryonic stem cells (ESC), or EK cells, to distinguish them from the teratocarcinoma-derived embryonal carcinoma (EC) cells (Solter & Damjanov, 1979; Martin, 1980). In this manuscript, embryo-derived pluripotent stem cells will be known as EK cells.

In certain culture conditions, it is possible for EK cells to differentiate into blood islands and even myocardium (Doetschman *et al.* 1985). The properties of EK cells could therefore be similar to normal embryonic cells in terms of their potential to recolonize haematologically deficient hosts.

The effectiveness of day-6 and -7 embryonic cells, obtained directly from the uterus, in the colonization of the haemopoietic system of recipient mice has been reported previously (Hollands, 1987). The present work aims to assess mouse embryonic growth *in vitro* and the ability of the resultant cells to colonize anaemic and X-irradiated recipients. The grafting of EK cells into recipients is also assessed.

Materials and methods

Blastocyst preparation and culture

Female C57Bl/10 mice were superovulated and mated as previously described (Fowler & Edwards, 1957). The day of finding the vaginal plug was taken as day 0 of gestation. On the third day of gestation, pregnant females were killed by cervical dislocation and their uteri removed and placed into

a sterile plastic culture dish, containing culture medium CMRL 1066 (Gibco Ltd). Each uterine horn was flushed with 1 ml of culture medium and the resulting blastocysts pooled into groups of 50 in drops of culture medium, under sterile paraffin oil (BDH Chemicals Ltd). All blastocysts were washed four times in medium CMRL 1066 to remove any contaminating maternal cells.

Medium CMRL 1066 was supplemented with 1 mM-glutamine (Flow Laboratories) and 1 mM-sodium pyruvate (Sigma Chemical Co.) for embryo culture. Heat-inactivated (56°C for 30 min) fetal calf serum (Sigma Chemical Co.) was used as a supplement, initially at a concentration of 10 %. After 2 days of culture, the concentration of fetal calf serum was raised to 20 %. Human umbilical cord serum was used instead of fetal calf serum, at the same concentrations, for comparison. Human cord blood was allowed to clot at 4°C, and then centrifuged at 1000 *g* for 30 min. The serum was then withdrawn, using a sterile Pasteur pipette, and heat inactivated at 56°C for 30 min. Human cord serum was then stored at −20°C until required. Samples older than 24 h when received and haemolysed serum were discarded. No antibiotics were used in the culture medium at any stage.

Blastocysts were cultured in 0·1 ml drops of media, under sterile paraffin oil, in groups of 50 or singly. Each 35 mm Primaria-coated culture dish (Falcon Plastics) contained four drops of culture media. Each dish therefore contained either 4 or 200 blastocysts. The cultures were maintained at 37°C in a humidified modular incubation chamber, (Billups-Rothenberg), containing 5 % CO_2 and 95 % air. Culture media were renewed daily with 0·1 ml of fresh supplemented media.

Harvesting of embryonic cells for grafting

Embryonic cells were harvested on day 3 and day 4 of culture. Since the blastocysts were day 3 when culture began, the harvesting times were equivalent to day 6 and 7 of gestation. The embryoid bodies, and occasional blood islands, were harvested by displacing the paraffin oil with excess culture media, and simple washing of the cells from the plate with phosphate-buffered saline. No enzyme treatment was used. Harvesting of cells by trypsinization results in a loss of colonizing ability (W. Reik, personal communication). Once harvested from the dish, the cells were centrifuged at 500 *g* for 5 min and then made up in 0·2 ml of phosphate-buffered saline for grafting. Each drop, containing 50 blastocysts, yielded $1{\cdot}1–2{\cdot}6\times10^4$ cells.

Preparation of EK cells

EK cells were prepared as described previously (Evans & Kaufman, 1981). Cells were harvested by trypsinization, and EK cells separated from fibroblast feeder cells by sedimentation. Separation was achieved by allowing the fibroblasts to settle for 2 h at unit gravity, and withdrawing the resultant supernatant which contains EK cells. The EK cells were then washed twice in phosphate-buffered saline. Between $0{\cdot}5–1{\cdot}0\times10^6$ EK cells were injected into each recipient.

Grafting of embryonic cells

Recipient mice came from two strains:

(1) Genotype 129-W^vW^v, obtained by crossing 129-W^v/+ heterozygotes (Department of Genetics, University of Cambridge). Recipients were grafted within 1–2 days of weaning, at an age of 21–23 days, and were male and female. The strain is not SPF and in our colony 90 % of untreated W^v homozygotes die within 2–3 days of weaning. 129-W^vW^v mice have chronic macrocytic anaemia due to a stem-cell deficiency (Russell & Bernstein, 1966).

(2) Balb/c (Olac Ltd), X-irradiated with 8·5 Gy, as previously described (Hollands, 1987).

All embryonic cells grown *in vitro*, and EK cells, were grafted by intravenous injection into the tail vein of the recipient. Cells remaining in the barrel of the syringe were used for cell counts. Control mice were injected with phosphate-buffered saline.

Analysis of grafted recipients

Blood samples were taken from recipients for electrophoresis, haematological assays and blood smears. The techniques for electrophoresis, densitometry and haematological assays were as previously described (Hollands, 1987).

Results

Embryonic growth in vitro

Day-3 blastocysts, kept in groups of 50 *in vitro*, expanded within 24 h and attached to the surface of the culture dish. Attachment was assessed by gently rocking the culture dish and observing the movement of the embryos. During this expansion and attachment phase, approximately 10 % of the blastocysts died. Dead blastocysts were removed from the culture dishes to minimize toxic effects from these degenerating embryos. Surviving blastocysts continued to grow, although normal embryonic morphology was lost. After attaching to the culture dish, the blastocysts collapsed and spread over the surface of the dish. Fibroblasts were not seen in significant numbers. Embryoid bodies were often formed in blastocysts grown for 3 and 4 days. Some of the blastocysts grown on for 4 days showed occasional aggregates of pigmented cells. These could have been blood islands. Normal embryonic development was not seen in any instance. Embryoid bodies and 'blood islands' were used as embryonic cell grafts.

Day-3 blastocysts, kept singly *in vitro*, died within 24 h of collection. None of these cells were used as embryonic cell grafts.

Fetal calf serum (FCS) and human cord serum (HCS) were compared and gave identical results when used as supplements to the culture medium. Since FCS is much easier to obtain than HCS, it was decided to use FCS in the main culture experiments.

Table 1. *Intravenous grafting of day-3 and day-4 cultured C57BL/10 blastocyst outgrowths into 129-*W^vW^v *anaemic mice*

Recipient number	No. of nucleated cells injected $\times 10^4$	Culture period of donor cells (days)	Donor Hb	Donor GPI	Survival postgraft (days)
1	4·9	3	+	+	320
2	5·3	3	+	+	355
3	6·1	3	+	+	306
4	5·6	3	−	−	2
5	6·3	3	+	+	360
6	7·0	3	+	+	311
7	5·8	3	+	+	347
8	6·5	3	+	+	315
9	7·1	3	+	+	351
10	5·7	3	+	+	317
11	6·8	4	+	+	309
12	8·1	4	+	+	319
13	8·4	4	+	+	322
14	9·1	4	+	+	331
15	8·9	4	+	+	329
16	9·5	4	−	−	2
17	9·7	4	+	+	312
18	10·0	4	+	+	335
19	10·5	4	+	+	300
20	10·8	4	+	+	341
21–30	0 (Saline)		−	−	2–3

All recipients died at the times shown.

Blastocysts grown for 3 and 4 days grafted into 129-W^vW^v *anaemic mice*

Blastocyst cultures from C57B1/10 mice at 3 and 4 days growth were harvested by simple washing, and injected intravenously into two groups of ten recipient 129-W^vW^v anaemic mice (Table 1). Within 24–48 h of injection with embryonic cells, 90 % of these animals were showing donor haemoglobin, representing approximately 10 % of the total, and rising in some recipients to 99 % by day 16 postgraft. Donor lymphocytic glucose phosphate isomerase was present in the same 90 % of recipients by 3–4 days postgraft, representing up to 10 % of the total initially, and rising to 99 % in some recipients within 16 days postgraft (Table 2). Donor markers appear to be completely stable in these animals. The remaining 10 % of recipients failed to colonize and died at the same time as controls. All of the successfully grafted recipients are now dead. Donor markers were present up to the time of death, and on autopsy no tumours were found.

Haematological assays on the successfully grafted animals showed a complete reversal of the anaemia after grafting (Table 3). Haemoglobin levels and erythrocyte counts returned to normal. The marked macrocytosis, characteristic of this anaemia, disappeared. Leucocyte counts did not change significantly in the postgraft period.

Control mice, injected with phosphate-buffered saline, died within 2–3 days of the injection. Cells obtained from blastocysts grown for less than 3 days *in vitro* were ineffective in colonizing any recipients.

Blastocysts grown for 3 and 4 days grafted into X-irradiated Balb/c mice

Blastocysts grown for 3 and 4 days *in vitro* were also capable of repopulating the haemopoietic system of two groups of ten lethally X-irradiated mice (Table 4). 65 % of recipient mice showed donor haemoglobin within 24–48 h postgraft at a level of 10 %. The same 65 % showed donor lymphocytic GPI within 3–4 days. Once established, these markers were stable. Approximately 10 % of successfully grafted X-irradiated recipients died from radiation damage of the gastrointestinal tract within 2·5 weeks postirradiation. These animals all suffered from severe diarrhoea before death. At the time of death, these recipients showed donor markers, indicating colonization by the donor embryonic cells. Death was apparently due to X-irradiation-induced gastrointestinal syndrome (Coggle, 1983) and not graft failure. All of the successfully grafted recipients are now dead. Donor markers were present up to the time of death, and on autopsy no tumours were found.

The haematological parameters were normal and stable in grafted mice (Table 5). Leucocyte counts remained steady, despite X-irradiation. Controls injected with phosphate-buffered saline died within

Table 2. *Percentage of donor C57Bl/10 haemoglobin and lymphocytic glucose phosphate isomerase in 129-W^{v}W^v recipients grafted with day-3 or -4 cultured blastocyst outgrowths (assessed by densitometry)*

Time postgraft (days)	Number of recipients with donor haemoglobin					
	0	1–10%	11–25%	26–50%	51–75%	76–100%
1	2	18				
2	0	10	5	3		
4	0	7	9	1	1	
8	0	3	2	1	12	
16	0	0	1	1	9	7
32	0	0	0	0	6	12
300	0	0	0	0	0	18
Time postgraft (days)	No. of recipients with donor lymphocytic GPI					
	0	1–10%	11–25%	26–50%	51–75%	76–100%
1	20					
2	18					
4	0	18				
8	0	9	6	2	1	
16	0	1	4	6	4	3
32	0	0	0	0	6	12
300	0	0	0	0	0	18

Table 3. *Haematological parameters of 129-W^{v}W^v anaemic mice grafted with day-3 or -4 cultured C57Bl/10 blastocyst outgrowths*

Time postgraft (days)	Haemoglobin ($g\,100\,ml^{-1}$) (Mean ± S.E.)	Erythrocyte count ($\times 10^9\,ml^{-1}$) (Mean ± S.E.)	Leucocyte count ($\times 10^6\,ml^{-1}$) (Mean ± S.E.)
Pregraft	8·0 ± 0·2	6·0 ± 0·2	3·5 ± 0·5
1	9·2 ± 0·1	7·1 ± 0·2	3·5 ± 0·2
7	11·0 ± 0·2	7·9 ± 0·2	4·1 ± 0·3
31	13·9 ± 0·1	8·1 ± 0·2	4·5 ± 0·2
200	14·6 ± 0·3	8·0 ± 0·2	4·6 ± 0·2
Normal mouse Controls	15·0 ± 0·2	8·0 ± 0·2	4·0 ± 0·2
1	7·0 ± 0·2	6·0 ± 0·1	3·2 ± 0·5
2	5·7 ± 0·1	3·1 ± 0·1	3·0 ± 0·6

12–14 days postirradiation, as in previous experiments (Hollands, 1987).

EK cells grafted into 129-W^{v}W^v anaemic and X-irradiated Balb/c mice

EK cells were incapable of colonizing the haemopoietic system of either 129-*W^{v}W^v* anaemic or X-irradiated Balb/c mice. Each mouse received an intravenous injection of $0{\cdot}5$–$1{\cdot}0\times10^6$ EK cells and was then assayed for the presence of donor type GPI. 20 X-irradiated Balb/c and 10 129-*W^{v}W^v* mice were given EK cells but in all cases the recipient mice died. 129-*W^{v}W^v* mice died within 2–3 days of the injection of EK cells, X-irradiated Balb/c mice died within 12–14 days of injection. These survival rates are the same as saline-injected control mice. The donor EK cell marker (GPI 1C) was not found in the blood of any recipient.

Discussion

Three main conclusions can be drawn from the present results on embryonic growth *in vitro*. First, embryonic haemopoietic stem cells can differentiate in an embryo which is otherwise developing abnormally. Second, maternal decidual cells can be excluded, without affecting the success of the graft. Last, pluripotent embryonic EK cells do not have the ability to colonize deficient recipients, when prepared using the present techniques.

It is surprising that blastocysts grown singly did not survive growth *in vitro*, whereas those grown in groups of 50 thrived. It is possible that the culture

Table 4. *Intravenous grafting of day 3 and day 4 cultured* C57B1/10 *blastocyst outgrowths into X-irradiated Balb/c mice*

Recipient number	No. of nucleated cells injected $\times 10^4$	Culture period of donor cells (days)	Donor Hb	Donor GPI	Survival postgraft (days)
1	5·1	3	+	+	315
2	4·8	3	+	+	322
3*	5·7	3	+	+	17
4	6·0	3	−	−	14
5	6·5	3	+	+	339
6	6·9	3	+	+	301
7	6·7	3	−	−	13
8	6·0	3	+	+	323
9	6·0	3	+	+	341
10	7·1	3	−	−	14
11	7·8	4	+	+	326
12	8·6	4	+	+	335
13	9·0	4	−	−	12
14	9·3	4	+	+	343
15*	8·8	4	+	+	18
16	9·8	4	+	+	311
17	9·9	4	−	−	12
18	10·1	4	+	+	326
19	10·3	4	−	−	14
20	10·6	4	+	+	13
21–30	0 (Saline)		−	−	12–14

Recipients marked '*' died at the time shown of X-irradiation damage of the gastrointestinal tract.

Table 5. *Haematological parameters of X-irradiated Balb/c mice grafted with day-3 or -4 cultured C57B1/10 blastocyst outgrowths, and control X-irradiated mice given saline*

Time postgraft (days)	Haemoglobin ($g\,100\,ml^{-1}$) (Mean ± S.E.)	Erythrocyte count ($\times 10^9\,ml^{-1}$) (Mean ± S.E.)	Leucocyte count ($\times 10^6\,ml^{-1}$) (Mean ± S.E.)
1	13·3 ± 0·1	7·0 ± 0·2	3·8 ± 0·2
7	14·7 ± 0·1	7·8 ± 0·2	4·0 ± 0·2
31	15·3 ± 0·1	8·7 ± 0·2	4·3 ± 0·2
62	15·2 ± 0·1	8·0 ± 0·2	4·0 ± 0·2
200	15·0 ± 0·1	8·2 ± 0·2	4·1 ± 0·2
Control mice			
1	13·0 ± 0·1	7·7 ± 0·2	2·6 ± 0·2
2	12·3 ± 0·1	7·0 ± 0·2	2·0 ± 0·2
4	11·3 ± 0·1	7·0 ± 0·2	0·9 ± 0·2
8	10·0 ± 0·1	6·5 ± 0·2	0·1 ± 0·02
12	4·8 ± 0·1	1·1 ± 0·2	0·03 ± 0·01

conditions caused the death of singleton embryos. Future development of the culture systems should allow the analysis of singletons grown *in vitro*.

The observation that haemopoietic cells can differentiate in abnormal embryos correlates with previous work which describes the formation of blood islands from embryos grown *in vitro* (Cole *et al.* 1966; Chen & Hsu, 1979). These workers were interested in the morphology of the cells obtained and did not attempt to use any of the blood island cells for grafting.

The present data show that embryonic cells obtained from embryos grown *in vitro* can colonize deficient recipients with a high efficiency. The presence of blood islands was not a prerequisite for success of the graft. Blastocysts grown for 3 days *in vitro* did not have any blood islands, only cystic embryoid bodies. Nevertheless, these cells were capable of colonizing recipients and, therefore, must include embryonic haemopoietic stem cells or their precursors. Embryonic haemopoietic cells are evidently capable of continued differentiation *in vivo*.

No tumours were found in recipients of embryonic cells grown *in vitro*. This suggests that embryonic cells do not have the same properties as teratocarcinomas

(Ishikawa, 1978), which induce tumour colonies in the lung when injected intravenously.

The data also show that maternal decidual cells are not required for the successful colonization of recipients. This observation has been reported before, (Hollands, 1987), when embryos were dissected free of decidua. The decidual cells can never be totally removed from the embryo by that method, some contamination would always occur. In the present experiments, decidual cells are excluded. Blastocysts were carefully washed on collection, which removes any maternal cells present. Maternal decidual cells cannot therefore be involved in the haemopoietic reconstitution of the recipients.

The rate at which embryonic cells grown *in vitro* colonize 129-W^vW^v and X-irradiated recipients, was slightly slower than embryonic cells obtained directly from the uterus. This conclusion is drawn from the contribution of donor haemoglobin at 24 h being 10 %, compared to 20 % in recipients of day-7 cells directly from the uterus (Hollands, 1987). The donor cells need to have a cell cycle of 1·6 h to produce this colonization in 24 h. This cell cycle agrees with that calculated for cells obtained directly from the uterus (Hollands, 1987), however, it is still difficult to rationalize. Further experiments are planned to assess fully this observation.

The minimum number of cells required for a successful graft is apparently lower when using donor cells derived from embryos grown *in vitro*, compared to cells obtained directly from the uterus. In 129-W^vW^v anaemic mice, the minimum successful dose was $4{\cdot}9\times10^4$ and, in X-irradiated Balb/c, $4{\cdot}8\times10^4$. It is difficult to say exactly how many blastocysts these cells originate from, although 200 were in each plate and the death rate was about 10 %. The minimum number of cells required for a successful graft, when using donor cells isolated directly from the uterus, was 8×10^5 (Hollands, 1987). The difference may represent the decidual cells. Nevertheless, colonization was achieved with one embryo directly from the uterus, but required approximately 180 embryos cultured *in vitro*. Culture *in vitro* is evidently detrimental to the development and differentiation of embryonic haemopoietic cells.

The leucocyte counts of grafted recipients remained constant throughout the postgraft period. The number of leucocytes should have decreased, especially in X-irradiated recipients. The source of these leucocytes requires further investigation and analysis.

It is surprising that EK cells were unable to colonize the haemopoietic system of any recipients. The preparation of EK cells for grafting requires trypsinization of the cells from the culture dish. It is possible that this trypsinization results in a loss of colonization ability. Cells obtained directly from blastocysts lose the ability to colonize when treated with trypsin. Improved harvesting techniques for EK cells, which do not require enzyme treatment, need to be developed in the future.

The present experiments are unable to yield any data on the possible colonization of myocardium by EK cells. Since none of the recipients had known myocardial deficiencies, colonization is unlikely. Nevertheless, this is an important area which requires assessment in the future.

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